Quantification of Two Aromatic Amine Mutagens, PBTA-1 and PBTA-2, in the Yodo River System

Takeshi Ohe,[1] Nobue Takeuchi,[1] Tetsushi Watanabe,[2] Atsuko Tada,[3] Haruo Nukaya,[4] Yoshiyasu Terao,[5] Hiroyuki Sawanishi,[6] Teruhisa Hirayama,[2] Takashi Sugimura,[3] and Keiji Wakabayashi[3]

[1]Department of Food and Nutrition Science, Kyoto Women's University, Kitahiyoshi-cho, Imakumano, Higashiyama-ku, Kyoto, Japan; [2]Department of Public Health, Kyoto Pharmaceutical University, 5-Nakauchi-cho, Misasagi, Yamashina-ku, Kyoto, Japan; [3]Cancer Prevention Division, National Cancer Center Research Institute, Chuo-ku, Tokyo, Japan; [4]School of Pharmaceutical Science; [5]Graduate School of Nutrition and Environmental Sciences, University of Shizuoka, Yada, Shizuoka, Japan; [6]Faculty of Pharmaceutical Sciences, Hokuriku University, Kanagawa-machi, Kanazawa, Japan

The levels of two aromatic amine mutagens, 2-[2-(acetylamino)-4-[bis(2-methoxyethyl)amino]-5-methoxyphenyl]-5-amino-7-bromo-4-chloro-2*H*-benzotriazole (PBTA-1) and 2-[2-(acetylamino-4-[*N*-(2-cyanoethyl)ethylamino]-5-methoxyphenyl]-5-amino-7-bromo-4-chloro-2*H*-benzotriazole (PBTA-2), were quantitatively analyzed in the Yodo River system in Japan. The river water samples were collected at nine sampling sites from the Yodo River system twice or three times between May and July in 1997. PBTA-1 and PBTA-2 in the river water samples were concentrated on blue rayon columns, partially purified by high-performance liquid chromatography (HPLC) on reverse-phase columns, then quantified by HPLC with an electrochemical detector. The amounts of PBTA-1 and PBTA-2 in the water samples were < 0.01–1.91 and < 0.01–2.25 ng/L, respectively. High levels of PBTA-1 and PBTA-2 were detected in the samples collected within 4 km downstream of two sewage plants, which are located along the banks of the Nishitakase River, a tributary of the Yodo River system, and these samples showed stronger mutagenicity in *Salmonella typhimurium* YG1024 with S9 mix than the other water samples. On the other hand, the river water samples from upstream of the sewage plant were weakly or not mutagenic and PBTA-1 and PBTA-2 were not detected. These results confirmed that a major source of PBTA-1 and PBTA-2 in the Yodo River system is effluent from the sewage plants and that discharged mutagens, including PBTA-1 and PBTA-2, are diluted and/or decomposed while moving down the Yodo River system. *Key words*: mutagenicity, PBTA-1, PBTA-2, quantification, river water. *Environ Health Perspect* 107:701–704 (1999). [Online 27 July 1999]
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Recent research has stated that a wide range of genotoxic compounds are released into river water from industrial, agricultural, and domestic sources (*1–3*), and that contaminated river water has been used as a supply of drinking water in some cases. Therefore, the analysis of mutagens in river water is of great importance from the standpoint of safety of the drinking water and the ecogenotoxicity of organisms in the water environment. However, the quantitative determination of mutagens in river water has remained unexplored. Water samples from the Yodo River system, which flows through the prefectures of Kyoto and Osaka in Japan, can be mutagenic to *Salmonella typhimurium* strain TA 98 with S9 mix. The major possible source of the mutagenicity is possibly the effluents from the two sewage plants along the bank of the Nishitakase River, a tributary of the Yodo River system, in Kyoto City (*4–8*).

In our previous studies (*9–11*), five mutagenic compounds were isolated from water samples taken at sites below the sewage plants of the Nishitakase River. The structures of two mutagens were determined to be 2-[2-(acetylamino)-4-[bis(2-methoxyethyl)-amino]-5-methoxyphenyl]-5-amino-7-bromo-4-chloro-2*H*-benzotriazole (PBTA-1) and 2-[2-(acetylamino)-4-[*N*-(2-cyanoethyl) ethylamino]-5-methoxyphenyl]-5-amino-7-bromo-4-chloro-2*H*-benzotriazole (PBTA-2), as shown in Figure 1. These mutagens are presumedly formed from azo dyes that are used as an industrial material for dyeing (*9–11*).

To clarify the distribution of PBTA-1 and PBTA-2 in the Yodo River system in the present study, the levels of these two mutagens in river water samples were collected at various locations of the Yodo River system and quantitatively determined. In addition, the mutagenicity of the river water samples and the ratio of contribution of PBTA-1 and PBTA-2 to the total mutagenicity of the blue rayon adsorbed materials were examined.

Materials and Methods

Materials. PBTA-1 and PBTA-2 were synthesized according to methods described previously (*10,11*). Blue rayon was obtained from Funakoshi Pharmaceutical Co., Ltd. (Tokyo, Japan). High-performance liquid chromatography (HPLC) grade methanol and acetonitrile were obtained from Wako Pure Chemical Industries (Osaka, Japan). All other chemicals and reagents were of analytical grade.

Collection and blue rayon treatment of river water samples. Water samples (40 L) were collected at each of the nine locations shown in Figure 2 on 13 May, 27 May, and 24 July 1997. Each water sample was passed through a glass column (25 mm × 300 mm) with 2 g blue rayon at the flow rate of 50 mL/min at room temperature. After passing the water sample through the column, the blue rayon was washed with 200 mL each of distilled water several times and excess water was absorbed onto paper towels. Adsorbed materials were then extracted by shaking the blue rayon in 100, 100, and 50 mL, respectively, of methanol/ammonia water (50:1, v/v) three times for 20 min each, as previously reported (*6*). The extracts were combined and evaporated to dryness, and the residue was dissolved in 400 µL of methanol.

Purification and quantification of PBTA-1 and PBTA-2 by HPLC. The residue dissolved in methanol was fractionated by HPLC using a Nanospace SI-1 chromatograph (Shiseido, Tokyo, Japan) on a semipreparative TSK-GEL ODS-120A column (10 µm particle size, 7.8 × 300 mm; Tosoh Corp., Tokyo, Japan). The mobile phase of 75% methanol was pumped in isocratically at a flow rate of 1.6 mL/min at ambient temperature, and the fractions corresponding to PBTA-1 and PBTA-2 were collected. The eluates were monitored for absorbance at 260 nm with an ultraviolet (UV) absorption spectra detector (Shimadzu SPD-10A, Kyoto, Japan). The eluted solution of these fractions was then evaporated to dryness. The residue was dissolved in 400 µL of 50% methanol and further purified with a reverse-phase CAPCELL PAK C_{18} (UG80, 5 µm, 4.6 × 150 mm; Shiseido). A mobile phase of 40% acetonitrile in 25 mM H_3PO_4/Na_2HPO_4 (pH 6.5) was pumped in at a flow rate of 0.8 mL/min at 35°C. The absorption of the eluate at 260 nm was monitored and the fractions corresponding

Address correspondence to T. Ohe, Department of Food and Nutrition Science, Kyoto Women's University, Kitahiyoshi-cho, Imakumano, Higashiyama-ku, Kyoto 605-8501 Japan. Telephone: 075 531 7155. Fax: 075 531 7216. E-mail: oeosaka@oak.ocn.ne.jp

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to PBTA-1 and PBTA-2 were separately collected and evaporated to dryness and then dissolved again in 400 µL of 50% methanol. The dissolved fractions were finally analyzed on a reverse-phase YMC-Pack ODS-A column (5 µm, 4.6 × 150 mm; YMC Co., Ltd., Kyoto, Japan) with an electrochemical detector (900 mV; Irica Σ985, Kyoto, Japan). The mobile phase of 40% acetonitrile in 25 mM H_3PO_4/Na_2HPO_4 (pH 2.0) was pumped in at a flow rate of 0.8 mL/min at 35°C.

Spectrophotometric analysis. UV absorption spectra of the fractions finally purified with HPLC on a YMC-Pack ODS-A column were recorded with a photodiode-array detector (Shiseido).

Mutation test. The mutagenicity assay was carried out by the preincubation method (*12*) on *S. typhimurium* YG1024 (T. Nohmi, National Institute of Health Sciences, Tokyo, Japan) in the presence of S9 mix. The S9 mix used in this study contained 50 µL S9 (phenobarbital- and 5,6-benzoflavone-induced rat liver S9) per 500 µL. Mutagenic activities of samples were calculated from linear portions of the dose–response curves, which were obtained with five doses with duplicate plates.

Results

The river water mutagens collected with blue rayon columns were separated by HPLC on a reverse-phase TSK-GEL ODS column at the first purification step. The fractions corresponding to authentic PBTA-1 and PBTA-2 with retention times of 35–40 min and 27–32 min, respectively, were separately collected. These fractions were further purified by a reverse-phase CAPCELL PAK C_{18} column. Typical chromatograms at the second purification step are shown in Figure 3. The peak fractions with the same retention times as those of authentic PBTA-1 (23–25 min) and PBTA-2 (30–32 min) were collected. These fractions were finally applied to an analytical YMC ODS-A column with an electrochemical detector. As shown in Figure 4, peaks corresponding to PBTA-1 and PBTA-2 were clearly detected at retention times of 15.5 and 32.7 min, respectively, and these peaks were further confirmed by their UV absorption spectra with a photodiode-array detector. Figure 5 shows a typical example of the UV absorption spectrum of the peak fraction coinciding with the retention time of PBTA-2 in the water sample: the retention time was identical to that of the authentic PBTA-2. The peak fraction corresponding to PBTA-1 from the water samples also showed the identical UV absorption spectrum to that of authentic PBTA-1 (data not shown). The recoveries of PBTA-1 and PBTA-2 during the purification processes were examined by addition of authentic PBTA-1 and PBTA-2 to the sample at levels similar to those detected in river water samples (5 ng each per liter), and were 52 and 56%, respectively, for PBTA-1 and PBTA-2. The minimum detection limit for these two mutagens was 0.01 ng/L of river water with an electrochemical detector.

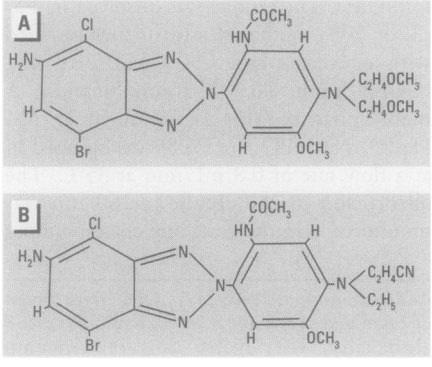

Figure 1. Structure of (*A*) PBTA-1 and (*B*) PBTA-2. Abbreviations: PBTA-1, 2-[2-(acetylamino)-4-[bis(2-methoxyethyl)amino]-5-methoxyphenyl]-5-amino-7-bromo-4-chloro-2*H*-benzotriazole; PBTA-2, 2-[2-(acetylamino)-4-[N-(2-cyanoethyl)ethylamino]-5-methoxyphenyl]-5-amino-7-bromo-4-chloro-2*H*-benzotriazole.

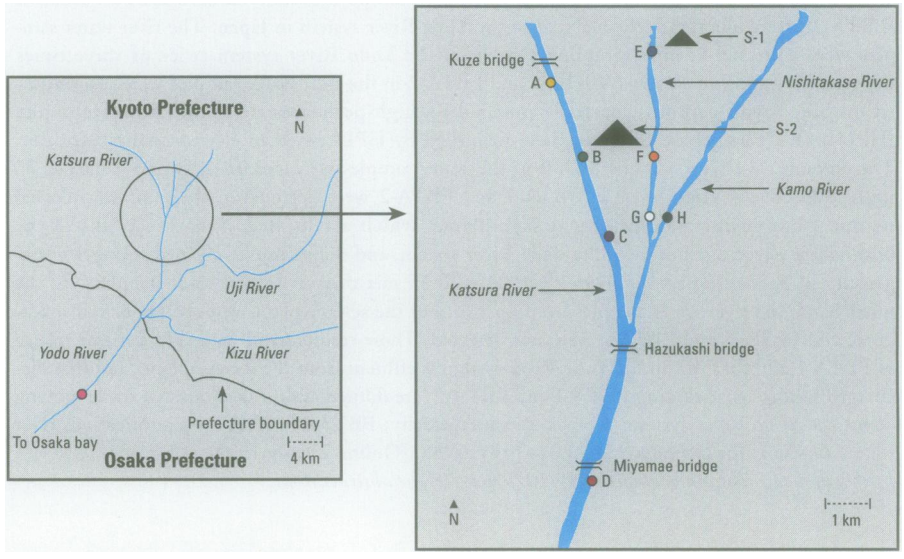

Figure 2. Sampling locations in the Yodo River system in Japan. S-1 and S-2 are sewage plants. Sampling sites are noted A–I.

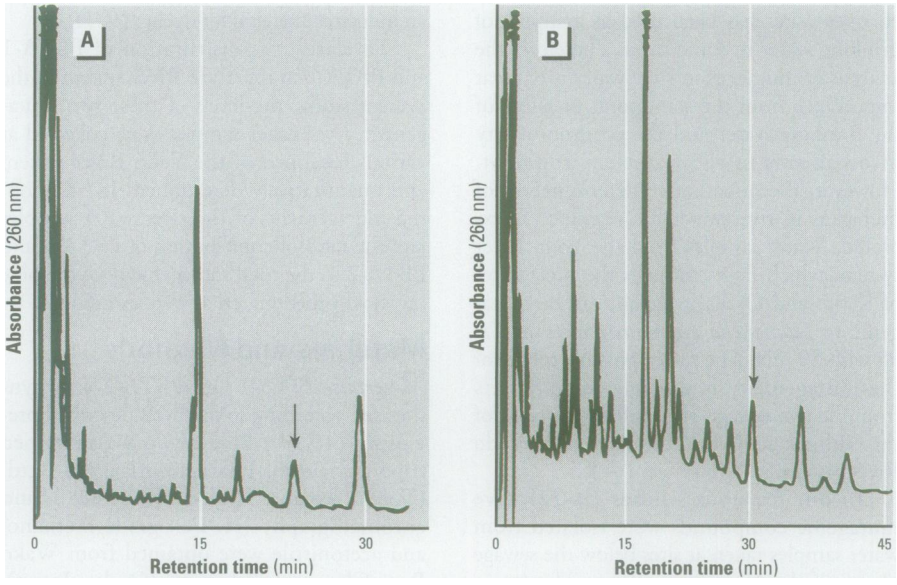

Figure 3. High-performance liquid chromatographic profiles of the second purification step. Abbreviations: PBTA-1, 2-[2-(acetylamino)-4-[bis(2-methoxyethyl)amino]-5-methoxyphenyl]-5-amino-7-bromo-4-chloro-2*H*-benzotriazole; PBTA-2, 2-[2-(acetylamino)-4-[*N*-(2-cyanoethyl)ethylamino]-5-methoxyphenyl]-5-amino-7-bromo-4-chloro-2*H*-benzotriazole. (*A*) PBTA-1 and (*B*) PBTA-2 in a river water sample from site E on 13 May 1997 on a CAPCELL PAK C_{18} column (Shiseido, Tokyo, Japan) were monitored by their absorbance at 260 nm. Injection volume: 14 L original river water.

Table 1 summarizes the amounts of PBTA-1 and PBTA-2 corrected for the recoveries of the compounds during the purification processes in the river water samples at the nine sampling sites shown in Figure 2. At all the sampling sites except for site B, water samples were collected three times: 13 May, 27 May, and 24 July 1997. At site B, the sampling was performed on 27 May and 24 July 1997. As shown in Table 1, PBTA-1 and PBTA-2 were detected in almost all the samples collected at sites B, C, and E–G, which are located within 4 km downstream of the sewage plants S-1 and S-2. The highest levels of PBTA-1 and PBTA-2 were observed for the sample collected at site E (1.91 ng/L for PBTA-1 and 2.25 ng/L for PBTA-2) in the Nishitakase River on 13 May 1997. The amounts of PBTA-2 in the samples collected at site B in the Katsura river on 27 May and 24 July 1997 were also high. Sites B and E are the nearest sites downstream from the sewage plants S-2 and S-1, respectively. PBTA-1 and PBTA-2 were not detectable in the samples collected on the 3 collection days upstream of S-2, at site A in the Katsura River, and at site H in the Kamo River. The amounts of PBTA-1 and PBTA-2 in the water samples collected at site I were smaller than those from site D, which is 12 km upstream of site I.

Mutagenicity of the river water samples toward *S. typhimurium* YG1024 with S9 mix and the ratio of contribution of PBTA-1 and PBTA-2 to the total mutagenicity of the blue rayon-adsorbed materials are also shown in Table 1. All water samples collected at sites B–G, which are located downstream from the sewage plants S-1 and/or S-2, showed mutagenicity in YG1024 with S9 mix. Mutagenic activity of samples from site A, which is upstream of S-2, was weaker than those from the downstream of S-2 (sites B–D). These results were consistent with the levels of PBTA-1 and PBTA-2 detected in those river water samples. All samples collected at site H in the Kamo River were not mutagenic, and neither PBTA-1 nor PBTA-2 was detected in these samples. Considering the concentrations of both PBTA-1 and

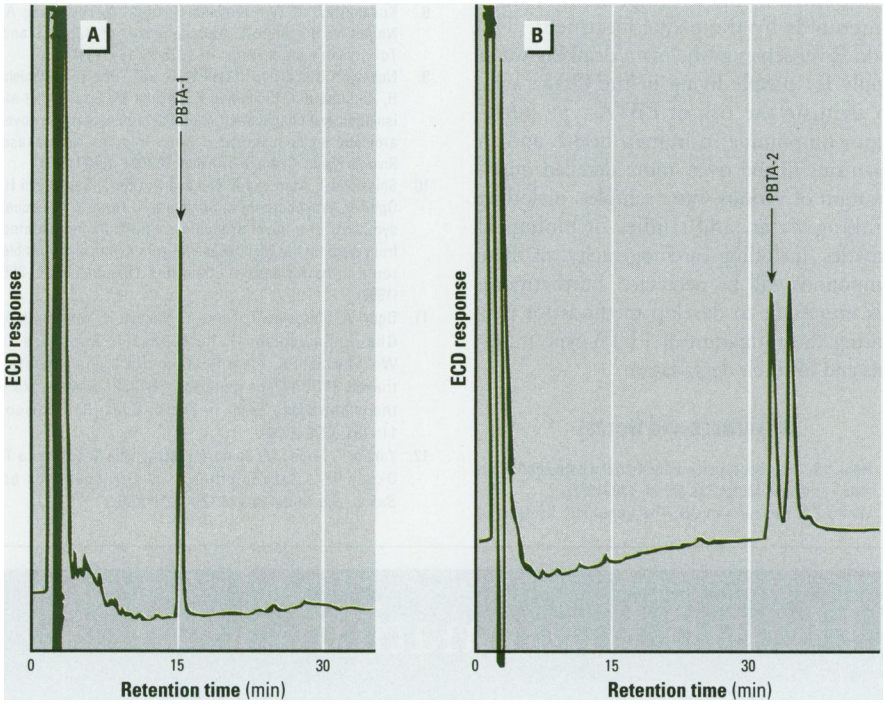

Figure 4. High-performance liquid chromatographic profiles of the final determination step. Abbreviations: PBTA-1, 2-[2-(acetylamino)-4-[bis(2-methoxyethyl)amino]-5-methoxyphenyl]-5-amino-7-bromo-4-chloro-2*H*-benzotriazole; PBTA-2, 2-[2-(acetylamino)-4-[*N*-(2-cyanoethyl)ethylamino]-5-methoxyphenyl]-5-amino-7-bromo-4-chloro-2*H*-benzotriazole. (*A*) PBTA-1 and (*B*) PBTA-2 in a river water sample from site E on 13 May 1997 on a YMC-Pack ODS-A column (YMC Co., Ltd., Kyoto, Japan) were monitored by the electrochemical detector response. Injection volume: 14 L original river water.

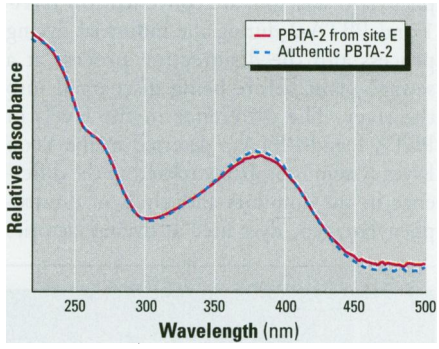

Figure 5. Ultraviolet absorption spectra of the peak fraction coinciding with the retention time of PBTA-2 in a river water sample. PBTA-2, 2-[2-(acetylamino)-4-[*N*-(2-cyanoethyl)ethylamino]-5-methoxyphenyl]-5-amino-7-bromo-4-chloro-2*H*-benzotriazole. A sample of the fraction containing approximately 20 ng of PBTA-2 from site E (taken on 13 May 1997) and authentic PBTA-2 were examined with a photodiode-array detector.

Table 1. Amounts of PBTA-1 and PBTA-2 and the ratio of contribution to mutagenicity of water samples in the Yodo River system.

River sampling site[a]	Concentration (ng/L)						Mutagenicity (revertants/L)			Contribution ratio (%)					
	PBTA-1			PBTA-2						PBTA-1			PBTA-2		
	13 May	27 May	24 July	13 May	27 May	24 July	13 May	27 May	24 July	13 May	27 May	24 July	13 May	27 May	24 July
Katsura															
A	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	900	0	800	0	0	0	0	0	0
B	[b]	0.47	0.27	[b]	0.92	0.82	[b]	4,400	26,600	[b]	9.5	0.9	[b]	16.3	2.4
C	0.08	0.02	0.37	0.50	0.06	0.83	18,600	2,200	29,000	0.4	0.8	1.1	2.1	2.1	2.2
D	< 0.01	0.16	0.17	0.03	0.13	0.28	9,600	1,500	15,000	0	9.4	1.0	0.2	6.7	1.5
Nishitakase															
E	1.91	0.54	0.22	2.25	0.37	< 0.01	37,500	11,300	2,100	4.5	4.2	9.3	4.7	2.5	0
F	1.09	1.09	0.03	1.18	0.21	0.23	40,300	4,700	15,500	2.4	20.5	0.2	2.3	3.5	1.2
G	0.62	0.29	0.08	0.96	0.14	0.12	57,900	1,900	15,500	0.9	13.5	0.5	1.3	5.7	0.6
Kamo															
H	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	0	0	0	0	0	0	0	0	0
Yodo															
I	< 0.01	0.03	< 0.01	< 0.01	0.14	0.05	4,600	0	2,400	0	[b]	0	0	[b]	1.6

Abbreviations: PBTA-1, 2-[2-(acetylamino)-4-[bis(2-methoxyethyl)amino]-5-methoxyphenyl]-5-amino-7-bromo-4-chloro-2*H*-benzotriazole; PBTA-2, 2-[2-(acetylamino)-4-[*N*-(2-cyanoethyl)ethylamino]-5-methoxyphenyl]-5-amino-7-bromo-4-chloro-2*H*-benzotriazole.
[a]Sampling was performed on 13 May, 27 May, and 24 July 1997. [b]Not tested.

PBTA-2 and their mutagenic potencies, these two compounds together accounted for < 10% of the total mutagenic activity of the river water, except for a few samples.

Discussion

The present study demonstrated that PBTA-1 and PBTA-2 are discharged from the sewage plants (S-1 and S-2) into the Katsura and/or the Nishitakse Rivers, and these compounds are diluted or decomposed while moving down the Yodo River system. We previously reported that PBTA-1 and PBTA-2 were synthesized from the corresponding dinitrophenylazo-type dyes, which are used for industrial purposes, by reduction with sodium hydrosulfite followed by chlorination with sodium hypochlorite (*10,11*). Sodium hydrosulfite is generally used in dyeing factories for discharge printing and bleaching. Sodium hypochlorite is mainly used in sewage plants for disinfection purposes. From these observations, it is most likely that the dyes, which have a dinitrophenylazo skeleton, are converted to PBTA-1 and PBTA-2 during the industrial dyeing process and the disinfection process at a sewage plant before being discharged into the river. The difference in the levels of PBTA-1 and PBTA-2 detected in the Yodo River system is probably related to the difference in the amounts and kinds of dinitrophenylazo-type dyes used at dyeing factories in Kyoto City. Because various kinds of dinitrophenylazo-type dyes are used as industrial materials, other PBTA-type compounds besides PBTA-1 and PBTA-2 would also contribute to the mutagenicity of the river water samples.

The amounts of PBTA-1 and PBTA-2 in the sample collected at site E on 24 July were lower than those collected on 13 May. Because ozone treatment apparatus was introduced into the sewage plant S-1 to treat sewage at the end of May 1997, the decrease in the PBTA-1 and PBTA-2 levels found at site E during the experimental period would be attributed to the decomposition of these compounds by the ozone treatment. The Yodo River serves as the main drinking water supply for people living in the Osaka area. To estimate the risk of PBTA-type mutagenic compounds to human health and to organisms in the river, more detailed quantification of various water samples, including drinking water, and studies of biological activities, including carcinogenicity, of these compounds will be necessary. Furthermore, it is important to develop methods for preventing the formation of PBTA-type mutagens and for their degradation.

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